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Prevalence and Associated Factors to Human Papillomaviruses Types 16 and 18 among Malagasy Women in Fianarantsoa, Madagascar

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Abstract In this study, we analyzed by means of polymerase chain reaction (PCR) cervical specimens obtained from consenting women in Fianarantsoa, 1712 cervical specimens obtained from them. The data obtained were compiled to establish correlation and prevalence of HPV. Different PCR techniques were used to detect HPV infection. Consensus primer sets MY and GP, and specific primers for HPV 16 and 18 were utilized to obtain the HPV typing. The result showed that 43 specimens are positive by MY and GP that 22(51.16%) are HPV 16 and 15(34.88%) HPV18. 67.56% of the infected are about 45 year old; 48.65% divorced; each woman had at least 2 different partners before marrying and more of 4 partners after divorce. 29.73% of them are married and everyone had at least 4 partners before marrying. 21.62% are unmarried, with multiple partners. Our results indicated that HPV infections, especially those HPV 16 and 18 represent a significant public health in Madagascar.

Keyswords Consensus Primers, Fianarantsoa, Human Papillomavirus (HPV), Madagascar, Polymerase Chain Reaction (PCR)

1. Introduction

The cervical cancer is the second most frequent cancer of the woman in the world[1]. Every year, close to 500'000 new cases of cervical cancer diagnosed are at the origin of 250'000 deaths[1]. About 80% of these cases occur in the developing countries, in particular in Latin America and in sub-Saharan Africa, where the cervical cancer occupies the first place[2]. Many factors of risk are bound to the apparition of this cancer, as the multiplicity of the sexual partners, the forwardness of the sexual intercourse and the first pregnancy, the low socioeconomic standard of living, the immune deficit or the tobacco addiction[3]. It has been established that the viral stub to high risk as the HPV16 and the HPV18 are the etiologic agent of the cervical cancer[4]. However, a lot of women infected by HPV viruses to high risk are not going all to develop the cervical cancer[5] but to constitute the healthy carriers. In developing countries, the prophylactic vaccine is not accessible to the patients and otherwise, the access to the centers of basis care is practically impossible in several cases. Otherwise, when the patients come to consult the physicians, in general, the cancer Is already to the advanced stage[3]. The necessity of the vaccine's clarification and the survey of the HPV type's prevalence to high risk are primordial of a future research putting in place prophylactic or therapeutic vaccines againstthe cervical cancer.

In Madagascar, according to the statistics held by the oncology service of the CHU/HJRA in Antananarivo that was the unique center of oncology, the cervical cancer occupies the second place after the cancer of the breast at the woman with about 470 new cases per year (yearly report of activity 2011)[6]. Due to a lack of tracking, but also because of the population sociocultural factors (the modesty, the level of education, the taboo…), the major part of the cases is diagnosed to an advanced stage.

The retrospective survey on the cervical cancer observed from 1992 to 2002 from 23 908 withdrawals addressed to the Institute Pasteur of Madagascar (IPM) for exam anatomopathological and 12 605 cervical smears for cytological exam showed well that in pathological anatomy, on 4 136 cases of diagnosed cancer, 2 621 cases (63. 4%) have been observed at the woman. On the total of the confirmed feminine cancers, 687 cases (26.2%) have a cervical localization. Three-quarters of the cervical cancer diagnosed are invasive with a middle age of 48. 2 years at the time of the diagnosis. The cytology of tracking doesn't have its diagnosed part that 74 cases of cancer. The precancerous lesions of the collar were 274 from which 61% diagnosed by

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the cytology[7].

In spite of this strong cancer prevalence, little study has been led to Madagascar concerning the cervical cancer prevalence or even less the prevalence of the HPV to high risk in the cervical cancer. The factors of risks associated to the illness were not yet the subject of scientific survey[6].

Currently, a national program of tracking by the visual inspection method to the acetic acid and the test of Papanicolaou (Pap smear) associated to the cryosurgery have been put in work but the results are not even available.

Thus, this work will carry on the prevalence determination of HPV 16 and 18 to the women in rural environment of MatsiatraAmbony, Amoron'I Mania and Vatovavy Fitovinany in the province of Fianarantsoa using the conventional PCR skill.

The choice of these three regions is based on the one hand to the conditions of life of the women, and on the other hand the remoteness to the capital where is the unique national tracking center.

The hope of Gardasil[HPV quadrivalent (types 6, 11, 16 and 18) Vaccine Recombinant] could not be realized in developing countries like Madagascar if the problem of the groups to high risk is not controlled through epidemiological studies on a big scale.

Indeed, this survey that has been achieved in the province of Fianarantsoa (Madagascar) had for goal to search for the prevalence of HPV 16 and 18 by the molecular biology technique and the risk factors to the infection of the HPV in this region.

2. Materials and Methods

2.1. Study Population

Women from selected rural health centers in the former province of Fianarantsoa (Madagascar) constituted the cohort of this study. The first step in the method consisted of an information session about cervical cancer and HPV being offered in each health center community. Women having suspected something wrong in their cervix were asked to volunteer to participate in the study by filling out a questionnaire requesting her age, education, sexual history, marital status, and tobacco consumption habits. Women at the beginning of pregnancy (less than 2 months) were also accepted into the study. The consent form, the questionnaire and the information pamphlet were written in the Malagasy language to facilitate the communication. A sterile disposable speculum was used for the gynecological exam. Cervical exfoliated cells were collected from all subjects by sampling the ectocervix and the endocervix using a cytobrush. The head of the cytobrush was kept in 5ml sterile phosphate buffered saline (PBS, pH 7.2) tube and frozen at -20˚C until DNA extraction. MY09/MY11 and GP5+/GP6+ consensus primer sets mediated PCR, amplifying DNA fragments in the conserved L1 region of approximately 450bp and 150bp respectively. Further, type-specific PCR primer sets allow the identification of individual genotypes have been widely used to study the natural history of human papillomaviruses (HPVs)[8][9][10]. All the specimens and corresponding questionnaires were carried out to the molecular biology laboratory in Fianarantsoa for analysis.

2.2. DNA Preparation

DNA from cervical cell samples was isolated as described previously[11][12]. Briefly, cervical cell suspensions were collected and treated with proteinase K (100 μg/ml) in lysis buffer (10mM Tris-HCl; pH 7.5, 1mM EDTA, pH 7.9; 0.5% SDS) overnight at 37°C. Standard phenol-chloroform extraction and ethanol precipitation were used for DNA purification. Pelleted DNA was resuspended in 50μL of deionized sterile water and stored at –20°C until further analysis. In order to determine the quality and the quantity of isolated DNA, each DNA was analyzed by electrophoresis on 1% agarose gels stained with ethidium bromide and spectrophotometrically[13].

2.3. Polymerase Chain Reaction (PCR)

Each amplification reaction was carried out in a total volume of 20μL overlaid with one drop of mineral oil and contained 10mM Tris–HCl (pH 8.3), 50mM KCl, 0.25U Taq DNA-polymerase, and 100–200ng DNA. The concentration of dNTPs and $MgCl_2$ varied with each set of primers. Each PCR was carried out in DNA thermal cycler (Eppendorf).

The conditions and the number of denaturation–annealing –extension cycles were different with each set of primers.

Aliquots (10 μL) of each PCR product were resolved by electrophoresis in a 2% agarose gel stained with ethidium bromide. The amplified products were identified by UV irradiation of the gels, andphotographed by UVP TRANSILLUMINATOR (BIODOC-IT IMAG SYSTEM) (Figure 1 and Figure 2).

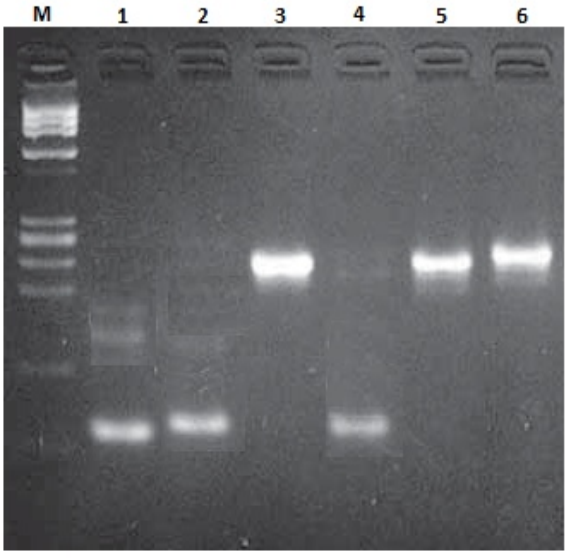

Figure 1. Electrophoresis of PCR products on 2% agarose gel (Image UVP Transilluminator, BIODOC-IT IMAG SYSTEM); **M**: DNA marker (ΦX174 DNA/HaeIII Marker). **(1) (2) (4)**: PCR with GP5+/GP6+ primers (142 bp), **(3) (5) (6)**: PCR with MY09/MY11 primers (451bp)

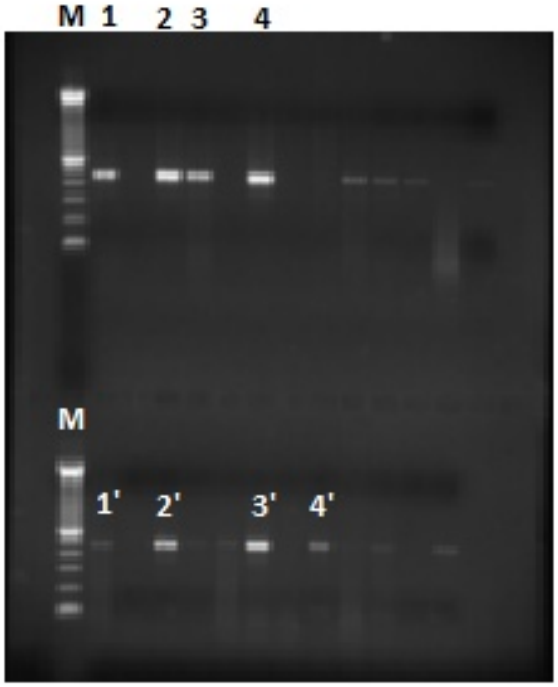

Figure 2. Electrophoresis of PCR products on 2% agarose gel (Image UVP Transilluminator, BIODOC-IT IMAG SYS); **M**: DNA marker (ΦX174 DNA/HaeIII Marker), HPV specific-primer directed PCR: **(1) (2) (3) (4)** PCR for HPV 16 (253 bp), **(1') (2') (3') (4')** PCR for HPV 18 (201 bp)

2.4. PCR Amplifications

Every samples have been respectively amplified with the MY and GP primers. The samples which give some lanes on agarose gel have been dealt with the type-specific primers for HPV types 16 and 18. All primers used in this study are shown in Table 1 and Table 2.

Table 1. Consensus primer sequences for HPV DNA detection

Primer	Sequence (5'-3')	Size (bp)	Reference
MY09 MY11	CGTCCMARRGGAWACTGATC GCMCAGGGWCATAAYAATGG	˜450 451 for HPV16	[17]
GP5+ GP6+	TTTGTTACTGTGGTAGATAC GAAAAATAAACTGTAAATCA	˜140 142 for HPV16	[18]

Table 2. Primer sequences for specific HPV types

HPV types	Primer sequence (5'-3')	Region	Size (bp)	Reference
16	CCCAGCTGTAATC ATGCATGGAGA GTGTGCCCATTAA CAGGTCTTCCA	E6/E7	253	[19]
18	CGACAGGAACGAC TCCAACGA GCTGGTAAATGTT GATGATTAACT	E6/E7	201	[19]

2.5. PCR with MY09 and MY11 Consensus Primers

The PCR mixture was complemented with 2.5 mM $MgCl_2$, 0.1 mM of each dNTP, 0.5 mM MY09 and MY11 primers.

The DNA amplification was carried out during 30 cycles which included the denaturation at 92°C for 30 s, the annealing at 53°C for 30 s, and the primer extension at 72°C for 30 s.

2.6. PCR with the GP5+ and GP6+ Consensus Primers

The PCR mixture was complemented with 2.5 mM $MgCl_2$, 0.05 mM of each dNTP and 0.5 mM of GP5+ and GP6+ primers. The DNA amplification was carried out during 40 cycles that included the denaturation at 94°C for 30 s, the annealing at 45°C for 30 s and the primer extension at 72°C for 30 s[14],[15].

2.7. PCR with the type-specific Primers for HPV types 16 and 18

The PCR mixture was complemented with 1.5 mM $MgCl_2$, 0.15 mM of each dNTP and 0.15 mM of each primer. The DNA amplification was carried out during 35 cycles that included the denaturation at 92°C for 15 s and the annealingand primer extension at 62°C for 4 min[16].

2.8. Statistical Analysis

The statistical analysis of the qualitative and quantitative variables was either of mono-varied type, or of multivariate type using a software Epinfo version 6. The Student test was used for the comparison of the averages and the variances and the test of Chi-2[8] for the comparison between the rates and a significant value if $p<0.05$.

3. Results and Discussions

The sensitization campaign for the free tracking of the HPV, 1712 women have been appropriated.

The average age of women appropriated is of 36.5 years (18 - 60) that means, in general, those women play a big role in the community and particularly, in the family. This number is meaningful in relation to the total number of the women in this region that Fianarantsoa is the second province the more populated of Madagascar.

This insufficiency to the number could be on the one hand, by the custom because according to the majority of the different ethnic groups in this region, the history of "sex" is reserved to the more aged women. On the other hand, this number would also especially be able to the impact of the insufficiency of the sanitary infrastructure in this region in rural environment that returns the population to get used from time to time to the traditional medicine.

After the test of HPV DNA by PCR using consensus primers in the 1712 specimens, 43 (2.51%) and 39 (2.27%) of specimens were respectively HPV positive in PCR using MY09/MY11 primers and GP5+/GP6+ primers ($p< 0.05$).

The result also show that the 39 positive in GP are the same specimens positive of the 43 tested by MY.

This difference can be translated that MY09/M11 primers are synthesized with several degenerate nucleotides in each primer and thus a mixture of 25 primers, capable of amplifying a wide range of HPV types[10]. The GP5+/GP6+ primers consist of fixed nucleotide sequence for each primer and detect a wide range of HPV types by using a lowered annealing temperature during PCR[13].Besides, the GP5+ and GP6+ primers are complementary to the part of the L1 region located inside the sequence recognized by the MY primers[18]. Indeed, the survey took consideration the results gotten with the MY primers.

HPV typing is of great importance for cervical cancer risk assessment, as well as planning individual treatment and vaccination strategies[15][16]. From those 43 specimens with positive result; we tested cervical specimens by PCR using type-specific primers HPV types 16 and 18. 22 (51.16%) are HPV 16; 15 (34. 88%) are HPV 18 and the remain are other types since MY09/MY11 and GP5+/GP6+ can detect HPV 6, 11, 16, 18, 31 and 33 that why there is a meaningful difference of the positivity rate between of HPV DNA by PCR using consensus and type-specific primers.

On the 37 infected by HPV 16 and HPV 18; 25 (67.56%) are about 45 years old and 12 (32.44%) are between 30 to 44 years old (p 0.05).

Questionnaires of every infected woman showed that 18 are divorced; each woman had at least 2 different partners before marrying and more of 4 partners after the divorce. Eleven of them are married and everyone had at least 4 partners before marrying. Eight are unmarried, they have multiple partners. The results also show that 29 on 37 chewing tobacco (powder) at least 4 times per day. Three smoke at least 2 cigarettes per day.

In this survey, the most elevated proportions of the HPV are diagnosed at the less aged women. These data coincide with those of Rakotondrajaona& al.[20] that find a middleage enters 41 and 48 years, but defer those of the other countries in development where the cervical cancer is considered like an illness of the women aged[21,22], or of Europe where the middle age is of 55 years[23]. This difference can explain itself by the slant of representativeness.

Even that there is not until now scientific proof showing the relation between the infection of HPV and partner's multiplicity, it is not necessary to disregard the high risk of the sexually transferable infection that encourages the infection of this virus.

The tobacco addiction increases the cervical cancer by the presence of the carcinogenic molecule (cotinine) a metabolite product of the nicotine in tobacco, acting as a cocarcinogen in cervical cells already infected by HPV 16 or 18 that had already the viral E6 and E7 oncogenes.

4. Conclusions

This survey confirms that the HPV infection exists at the Malagasy women and so that the survey is centred only on HPV16 and 18, the results well showed that the HPV 16 is the most frequent genotype. The majority of Malagasy women in rural environment took tobacco powder to help them all day for the hard work in the rice field.The analysis of the subject sexual partner number or her sexual partners was not conclusive to the effect on HPV infection in this survey.

From this survey, the feasibility in Madagascar of a tracking program of cervical cancer by molecular biology technique constitutes the essential question; the necessity of such exam is indisputable if its realization poses problems of practical application.

In perspective, the analysis of all different HPV typeshigh risk will be important in order to determine the types the more frequent in Madagascar.

Here are some recommendations made for the remainder of this research, and for future epidemiology studies in Madagascar: struggle against tobacco and analysis of other HPV genotypes.

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